(51) International Patent Classification 6 : A61K 38/43, 38/54, C12N 9/02, C12Q 1/00, 1/26, 1/68, C12P 1/00	A1	(11) International Publication Number: **WO 98/53849** (43) International Publication Date: 3 December 1998 (03.12.98)

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(54) Title: SYNTHETIC METALLOPROTEINS AND METHOD OF PREPARATION THEREOF

(57) Abstract

The subject invention provides synthetic metalloproteins containing at least one metal binding site consisting of a metal ion and ligands associated therewith. Such metalloproteins are prepared by using protein structure coordinate arrays to determine a set of primary sequence mutations in a preselected host protein, and constructing the synthetic metalloproteins by methods of recombinant genetics, protein expression and purification. Particular applications include synthetic superoxide dismutases and monooxidases. Also provided is a method of oxidizing an alkane using a synthetic metalloprotein in which a monooxygenase active site is installed. Other uses of synthetic metalloproteins include a method of treating a subject suffering from reperfusion type tissue damage and from ischemia.

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SYNTHETIC METALLOPROTEINS AND METHOD OF PREPARATION THEREOF

Cross-Reference To Related Applications

This application claims priority pursuant to 35 U.S.C. §119 based upon Provisional
Application Serial No. 60/047,777 filed May 27, 1997, the entire disclosure of which is
hereby incorporated by reference.

Field of the Invention

The present invention is in the field of synthetic metalloproteins, and relates to
compositions having the ability to undergo electron or atom transfer reactions. In
particular, the invention relates to proteins which are synthetically modified to incorporate
a redox-active metal site. The invention further relates to a method for preparing such
compositions, which are useful as catalysts in a broad range of applications, including, but
not limited to, hydrocarbon oxidation and superoxide dismutation reactions.

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Background of the Invention

Metalloproteins offer a particularly interesting target for the design of function, because the biological chemistry of metals is extraordinarily rich. Protein design methodologies (Bryson, J. W., *et al.*, (1995) *Science* **270**, 935-41; Desjarlais, J. R. &

5   Handel, T. M. (1995) *Curr. Opin. Biotechnol.* **6**, 460-6; Barrick, D. (1995) *Curr. Opin. Biotechnol.* **6**, 411-8) exist to incorporate transition metal ions into proteins (Barrick, *supra;* Higaki, J. N., Fletterick, R. J. & Craik, C. S. (1992) *Trends Biochem. Sci.* **17**, 100-4; Matthews, D. J. (1995) *Curr. Opin. Biotechnol.* **6**, 419-24; Regan, L. (1995) *Trends Biochem. Sci.* **20**, 280-5). A major goal of these systems is to exploit their catalytic or

10   electron transfer functions. However, prior to the present invention, there was no method of rationally engineering metalloproteins to introduce novel catalytic functions (Matthews, *supra;* Regan, *supra;* Tawfik, D. S., Eshhar, Z. & Green, B. S. (1994) *Mol. Biotechnol.* **1**, 87-103; Hellinga, H. W. (1996) in *Design of Metalloproteins*, eds. Cleland, J. L. & Craik, C. S. (Wiley-Liss, New York), Chapter 14, 369-398).

15   One of the present inventors used the rational protein design algorithm DEZYMER (Hellinga, H. W. & Richards, F. M. (1991) *J. Mol. Biol.* **222**, 763-85) in an attempt to design catalytically active metalloenzymes. The DEZYMER program was found capable of generating all chemically possible sites, but no teaching existed allowing the selection of functional metalloproteins. Common problems with the earlier work included the

20   inappropriate positioning of the active site and incorrect metallation of the protein at a location other than the intended active site. For the first time, it is possible to construct a five-coordinate non-heme iron active site analogous to that found in iron-dependent superoxide dismutase (Stoddard, B. L., *et al.*, (1990) *Biochemistry* **29**, 8885-93; Stallings, W. C., *et al.*, (1983) *Proc. Nat'l Acad. Sci. USA* **80**, 3884-8), as, for example, in the host

25   protein thioredoxin (Trx) (Figure 1). The construction of this site represents a challenge very different from the design of coordinatively saturated, enzymatically inert, metal sites (Regan, *supra;* Hellinga, *supra;* Coldren, C. D., Hellinga, H. & Caradonna, J. P. (1997) *Proc. Nat'l Acad. Sci. USA* **94**, 0000).

In contrast to previous protein design efforts, the present invention shows how to

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create a true active site which catalyzes the inner sphere transfer of electrons both to and from small molecule substrates. In order for this chemistry to occur, an open coordination position, or one occupied by a labile ligand such as H_2O, is required, but the site must be sufficiently buried to prevent protein dimerization via a metal bridge. Introduction of a

5   cavity for anion binding as well as other factors relating to the attraction and transport of the anion to the active site all must be considered as well.

The construction of a functional metal center requires that three sets of factors be taken into account: i) correct protein folding, ii) coordination requirements of the metal, iii) modulation of the properties of the metal center and protein matrix to achieve the

10   required control of reactivity. Previously, metalloprotein designs have focused primarily on the first two factors (Bryson, J. W., *supra*; Higaki, J. N., *supra*; Matthews, D. J., *supra*; Regan, L., *supra*; Tawfik, D. S., *supra*; Hellinga, H. W. (1996) *Curr. Opin. Biotechnol.*, 7, 437-441; Hellinga, H. W. (1996) in *Protein Engineering: Principles and Practice*, eds. Cleland, J. L. & Craik, C. S. (Wiley-Liss, New York), Chapter 14, pp 369-

15   398. Since the redox properties of {Fe_4S_4} centers are strongly dependent on the protein environment, the present inventors understood that this system would permit the modulation of the metal center by the protein (Johnson, M. K., *supra*; Stephens, P. J., Jollie, D. R. & Warshel, A. (1996) *Chem. Rev.* 96, 2491-2513).

Accordingly, the present inventors have applied their method *inter alia* to design

20   proteins containing cuboidal iron-sulfur clusters. Fe_4S_4 clusters are among the most common electron transfer centers found in biology (Johnson, M. K. (1994) in *Iron-Sulfur Proteins*, ed. King, R. B. (John Wiley & Sons, Oxford), Vol. 4, pp. 1896-1915). These clusters act as either simple soluble electron-transfer agents, membrane-bound components of electron-transfer chains, or parts of the electron reservoir found in complex

25   metalloenzymes in plants, animals and bacteria. In addition to participating in electron transport systems and in the metabolism of carbon, oxygen, hydrogen, sulfur and nitrogen, these centers also exhibit gene regulatory, catalytic and structural functions, and have been found as part of a morphogenetic protein (Johnson, M. K., *supra*).

In addition, the present inventors demonstrate herein methods having sufficient

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predictive power to redesign the hydrophobic interior of a protein (Trx) and thereby introduce an iron center that can catalyze the dismutation of superoxide anion. The present inventors also provide a method of preparing a HiPIP cluster constructed by placing a cuboidal $\{Fe_4S_4\}$ cluster in the hydrophobic interior of the protein thioredoxin

5   (Trx-Fe$_4$S$_4$).

Summary of the Invention

Accordingly, one object of the present invention is to provide a method of

10   preparing a functional metalloprotein containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion(s) and in a second coordination sphere, all atoms bonded directly and

15   indirectly to the atoms of the first coordination sphere between the metal ion(s) and the backbone of the protein, which comprises:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively

20   form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed apoprotein, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

25   (2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) identifying volume elements in the host protein excluding the backbone of the

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protein equal to between about 90% and 110% of the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating at least one set of primary sequence mutations;

(5) calculating the volume change resulting from each set of primary sequence mutations; and

(6) selecting a set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

(E) overexpressing the designed apoprotein in the host organism; and

(F) either (i) purifying the overexpressed designed apoprotein, and reconstituting it with the metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii) incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

Another object of the invention is to provide a functional metalloprotein containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and, in a second coordination sphere, all

the metal ion(s) and the backbone of the protein, which comprises:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively

5   form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed apoprotein, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array;

10   (2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) identifying volume elements in the host protein excluding the backbone of the protein equal to between about 90% and 110% of the volume of the metal binding

15   site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating at least one set of primary sequence mutations;

(5) calculating the volume change resulting from each set of primary sequence mutations; and

20   (6) selecting a set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed

25   apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

(E) overexpressing the designed apoprotein in the host organism; and

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(F) either (i) purifying the overexpressed designed apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially

5 denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii) incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

A further particular object of the present invention is to provide a synthetic
10 superoxide dismutase containing at least one metal binding site, which superoxide dismutase is useful in the treatment of a range of conditions, including, but not limited to, ischemia and reperfusion-type tissue damage.

An additional object of the present invention is to provide a method of oxidizing an alkane which comprises treating the alkane with a functional metalloprotein containing
15 a metal binding site wherein the metal binding site is selected from the group consisting of a $Mo(=O)_2$ complex and a mononuclear iron center together with a cubic iron-sulfur cluster under conditions suitable to form an oxidized alkane.

Another object of the invention is to provide a method of treating a subject suffering from ischemia which comprises administering to the subject a functional
20 metalloprotein prepared from a host protein containing a metal binding site wherein the protein has superoxide dismutase activity in a therapeutically effective amount.

A further application of the present invention is a method of treating a subject suffering from reperfusion type tissue damage which comprises administering to the subject a functional metalloprotein having superoxide dismutase activity.

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Brief Description of the Drawings

Figure 1 shows the location of the designed {Fe3+}Trx-SOD metal-binding site formed

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by Leu7His, Phe27Asp, Ile60His, and Asn63His. Other mutations include His6Asn (eliminates potential alternative metal binding residue), Cys32Ser and Cys35Ser (prevents disulfide formation), Leu58Ala and Thr66Ala (rebuilds complementary surface) and Asp26Ala (increases global stability of host protein).

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Figure 2 illustrates a 20% SDS gel of purified apo Trx-SOD. Lanes: 1, 28 µg of purified Trx-SOD; 5, 0.28 µg of purified Trx-SOD; 6, 0.78 µg of purified Trx-SOD; 2 and 3, molecular weight markers as indicated; 4, 1.8 µg of *E. coli* Fe SOD (Sigma).

10   Figure 3 provides a UV/vis spectrum of apo (solid) and reconstituted (dashed) Trx-SOD. The spectra of Trx-SOD (1.1×10^{-4} M) in 20 mM Hepes, pH 8.0, 25 mM NaCl, 21°C before and after iron reconstitution with ferrous ammonium sulfate in air are shown.

Figure 4 illustrates anion binding by a functional metalloprotein. The UV/vis spectrum
15   of $\{Fe^{3+}\}$Trx-SOD (1.1×10^{-4} M) in 20 mM Hepes, pH 8.0, 25 mM NaCl, 21 °C (solid line) was recorded. The sample was then adjusted to 100 mM sodium azide (dashed line, Figure 4A) or 100 mM sodium fluoride (dashed line, Figure 4B). Dotted lines represent difference spectra in both Figures.

20   Figure 5 provides an assay of $\{Fe^{3+}\}$Trx-SOD for SOD activity. Each point represents the average of three assays; the correlation coefficient of the least squares fit is greater than 0.99.

Figure 6 shows iron-limiting reconstitution and SOD activity. Apo Trx-SOD was
25   reconstituted in air to the following Fe:protein ratios: 0, 0.25, 0.50, 0.75, 0.95. The samples were then assayed for SOD activity (o) and the optical spectra (•) of the samples (20 µl diluted to 1 ml) were recorded. Each point represents the average of three assays; the correlation coefficient of the least squares fit is greater than 0.99.

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Figure 7 illustrates ribbon and space-filling representations of the designed cuboidal iron-sulfur protein Trx-Fe$_4$S$_4$. Iron is cyan, sulfur yellow. (*A*) Ribbon diagram showing the location of all mutations used in the construction of Trx-Fe$_4$S$_4$ and the region of the Trx host protein implicated in phage assembly. The cuboidal {Fe$_4$S$_4$} cluster binding residues

5   (Leu24Cys, Leu42Cys, Val55Cys, and Leu99Cys) are buried between the central β-sheet and two α-helices. Cys32Ser and Cys35Ser mutations were made to remove the native Trx disulfide bond, thereby eliminating any interaction from cysteine residues that are not part of the designed site. An isosteric Asp26Leu mutation was also introduced to improve the global stability of the protein; the Asp26Ala mutant of thioredoxin is stabilized by 3

10   kcal compared to the wild-type protein (Langsetmo, K., Fuchs, J. A. & Woodward, C. (1991) *Biochemistry* **30**, 7603-7609; Langsetmo, K., Fuchs, J. A., Woodward, C. & Sharp, K. A. (1991) *Biochemistry* **30**, 7609-7614). The residues that are believed to be important for protein-protein interactions in Trx function (Katti, S. K., LeMaster, D. M. & Eklund, H. (1990) *J. Mol. Biol.* **212**, 167-184; Dyson, H. J., Holmgren, A. & Wright,

15   P. E. (1989) *Biochemistry* **28**, 7074-7078; Dyson, H. J., *et al.*, (1990) *Biochemistry* **29**, 4129-4136; Russel, M. & Model, P. (1985) *Proc. Nat'l Acad. Sci., USA* **82**, 29-33) are colored red (Eklund, H., *et al.*, (1984) *EMBO J.* **3**, 1443-1449). (*B*) Space-filling representation of residues 24, 42, 55, and 99 in native *E. coli* Trx (left) and Trx-Fe$_4$S$_4$ (right). Incorporation of the {Fe$_4$S$_4$(Cys)$_4$}$^{n-}$ design moiety represents a conservative

20   isovolume exchange; there is only a negligible decrease in the volumes occupied by amino acid side-chains in the interior of the designed iron-sulfur protein relative to the host.

Figure 8 provides an optical spectrum of apo Trx-Fe$_4$S$_4$ (trace A), holo Trx-Fe$_4$S$_4$ (trace B) in 15 mM MOPS, 100 mM NaCl, pH 7.4, 5 °C, and the synthetic cluster {Fe$_4$S$_4$(S-

25   EtOH)$_4$}(Me$_4$N)$_2$ (trace C) in 10 mM CHES, 10 mM βME, pH 8.5. Holo-Trx-Fe$_4$S$_4$ contains 3.9 ± 0.2 moles of iron and 3.9 ± 0.2 moles acid labile sulfide per Trx protein; λ_{max} = 280 nm, $\epsilon_{M,280}$ = 36,000, λ_{max} = 413 nm, $\epsilon_{M,413}$ = 16,100. Inset: CD spectra of apo Trx-Fe$_4$S$_4$ (upper trace) and Trx (lower trace) in 50 mM potassium phosphate, pH

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7.0, 25 $^{\circ}$C. Upper trace has been offset slightly to clarify the close superposition of the two curves.

Figure 9 shows the stability of the free synthetic cluster $\{Fe_4S_4(S\text{-}EtOH)_4\}(Me_4N)_2$ ($\square$)
5   in the presence of low levels (0.4 mM) of stabilizing exogenous βME and reconstituted holo Trx-Fe_4S_4 ($\blacksquare$) in the absence of stabilizing exogenous βME. Solution conditions 10 mM MOPS, 100 mM NaCl, pH 7.4, 5 $^{\circ}$C.

Figure 10 provides an EPR spectrum of holo Trx-Fe_4S_4 in 15 mM MOPS, 100 mM NaCl,
10   pH 7.4, protein concentration 70±5 µM. Trace B: holo Trx-Fe_4S_4 oxidized with 10

equivalents of $K_3\{Fe(CN)_6\}$. Trace A: untreated (resting state) holo Trx-$Fe_4 S_4$. EPR

spectra collected on a Varian E-line EPR spectrometer operating at 9.24 GHz, 1.5 mW

observe power, 10 G modulation amplitude, 25 Kelvin.

15   **Figure 11** shows a set of consensus sequences found in proteins containing cuboidal {Fe4S4} clusters.

20   **Detailed Description of the Invention**

The present invention provides a rational design approach to construct a synthetic metalloprotein containing a metal center within a protein matrix of known structure. Accordingly, the present invention provides a method of preparing a functional metalloprotein containing at least one metal binding site, said site consisting of at least one
25   metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and in a second coordination sphere, all atoms bonded directly and indirectly to the atoms of the first

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coordination sphere between the metal ion(s) and the backbone of the protein, which comprises:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed apoprotein, wherein said determining comprises:

> (1) representing the structure of the host protein as a three-dimensional atomic coordinate array;

> (2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

> (3) identifying volume elements in the host protein excluding the backbone of the protein equal to between about 90% and 110% of the volume of the metal binding site;

> (4) combinatorially replacing sequence residues in said volume elements, thereby generating at least one set of primary sequence mutations;

> (5) calculating the volume change resulting from each set of primary sequence mutations; and

> (6) selecting a set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

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(E) overexpressing the designed apoprotein in the host organism; and

(F) either (i)   purifying the overexpressed designed apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii)   incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

In a certain embodiment, the invention provides a method as dislcosed above wherein the determining step comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination

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sphere overlap, such that if said radii overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein.

In a typical embodiment, the invention provides a method wherein the functional metalloprotein contains one metal binding site. The method is applicable in preparing functional metalloproteins with two, three or more metal binding (active) sites. In a particular embodiment, the method may be applied to prepare proteins wherein the metal binding site comprises a cuboidal iron-sulfur cluster. In another particular embodiment, the method may be used to prepare metalloproteins wherein the metal binding site comprises a mononuclear iron(II/III)-histidine. In the practice of the invention, the required primary sequence mutations may be determined using any molecular modeling protocol adapted to the purpose, for example, DEZYMER, PROPAK and METALSEARCH. Most commonly, the modeling method used in conjunction with the invention to determine the primary sequence mutations is any method based on the DEZYMER algorithm, as further described herein.

In order to use a modeling protocol, detailed structural information is required to

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provide a three-dimensional atomic coordinate array. Where crystals of the host protein are available, crystallographic data derived from X-ray crystallography, electron diffraction or neutron diffraction may be used. Coordinate arrays for protein crystals are readily obtained from public data banks such as the Brookhaven Protein Databank. In cases

5   where no crystals are available, or diffraction data are insufficient, and only a solution-phase structure determination is feasible, a three-dimensional atomic coordinate array may be derived by nuclear magnetic resonance spectroscopy.

The method of the present invention may be applied to a wide range of host proteins. For example, functional metalloproteins may be designed and constructed in

10   accord with the invention wherein the host protein is any physiological plasma protein, a thioredoxin or human serum albumin. Other proteins include catalytic antibodies, non-human serum albumins, such as bovine serum albumin, collagen, synthetic oligopeptides and polypeptides.

The present invention also provides a functional metalloprotein containing at least

15   one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and in a second coordination sphere, all atoms bonded directly and indirectly to the atoms of the first coordination sphere between the metal

20   ion(s) and the backbone of the protein, which comprises:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively form the first and second coordination spheres of the metal binding site and result in

25   overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed apoprotein, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array;

(2) defining the first and second coordination spheres of the metal binding site,

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comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) identifying volume elements in the host protein excluding the backbone of the protein equal to between about 90% and 110% of the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating a set of primary sequence mutations;

(5) calculating the volume change resulting from each set of primary sequence mutations;

(6) selecting the set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

(E) overexpressing the designed apoprotein in the host organism; and

(F) either (i) purifying the overexpressed designed apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii)   incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

In one embodiment, the invention provides a functional metalloprotein as disclosed above wherein the determining step comprises:

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(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

    (i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

    (ii) identifying residues to be repacked, comprising:

        a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii overlap, the residues are repacked; and

        b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume

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change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein.

Typically, the invention provides a functional metalloprotein wherein the protein contains one metal binding site. However, functional metalloproteins with two, three or more metal binding (active) sites are also available. One example of functional metalloprotein within the scope of the invention is a protein wherein the metal binding site comprises a cuboidal iron-sulfur cluster. Another example is a functional metalloprotein wherein the metal binding site comprises a mononuclear iron(II/III)-histidine. The functional metalloprotein provided by the invention may be prepared from a host protein which is a physiological plasma protein, a thioredoxin or a human serum albumin. The primary sequence mutations required in the process to prepare the functional metalloproteins may be determined using any molecular modeling protocol adapted to the purpose, such as the DEZYMER, PROPAK and METALSEARCH programs. The modeling method preferably used to determine the primary sequence mutations is any method based on the DEZYMER algorithm.

The present invention further provides a synthetic superoxide dismutase containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and in a second coordination sphere, all atoms bonded directly and indirectly to the atoms of the first coordination sphere between the metal ion(s) and the backbone of the synthetic superoxide dismutase, said synthetic superoxide dismutase being prepared by:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively

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form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed superoxide dismutase apoprotein, wherein said determining comprises:

5   (1) representing the structure of the host protein as a three-dimensional atomic coordinate array;

(2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

10   (3) identifying volume elements in the host protein excluding the backbone of the protein equal to between about 90% and 110% of the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating a set of primary sequence mutations;

15   (5) calculating the volume change resulting from each set of primary sequence mutations;

(6) selecting the set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting

20   metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed superoxide dismutase apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed superoxide dismutase apoprotein;

25   (D) transfecting a host organism with the nucleotide sequence of the designed superoxide dismutase apoprotein;

(E) overexpressing the designed superoxide dismutase apoprotein in the host organism; and

(F) either (i)   purifying the overexpressed designed superoxide dismutase apoprotein,

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and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed superoxide dismutase apoprotein, treating the partially denatured designed superoxide dismutase apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii)   incubating the metal ion(s) or metal cluster with the designed superoxide dismutase apoprotein and purifying directly an intact functional metalloprotein.

In one embodiment, the synthetic superoxide dismutase disclosed above is provided wherein the determining step comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination

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sphere overlap, such that if said radii overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting synthetic superoxide dismutase containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed synthetic superoxide dismutase apoprotein.

In another embodiment, the invention provides a synthetic superoxide dismutase wherein said superoxide dismutase contains one metal ion binding site. In other embodiments, the synthetic superoxide dismutase may contain two or more metal ion binding sites.

In the practice of the present invention, the primary sequence mutations necessary in the design of the superoxide dismutase are determined using any suitable modeling protocol, for example, DEZYMER, PROPAK and METALSEARCH, but preferably using the DEZYMER algorithm, as described below. In the design of the synthetic superoxide dismutase, the structure of the host protein is obtained as a three-dimensional atomic coordinate array from X-ray crystallography, electron diffraction or neutron diffraction where crystals are available for the host protein, or from nuclear magnetic resonance spectroscopy where no crystals have bene obtained.

Construction of the synthetic superoxide dismutase may be accomplished by

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selecting any of a variety of host proteins, such as thioredoxin or human serum albumin. Selection of other suitable host proteins in accord with criteria set forth herein would be within the level of skill of the ordinary worker.

The present invention also provides a synthetic monooxidase containing at least
5   one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and, in a second coordination sphere, all atoms bonded directly and indirectly to the atoms of the first coordination sphere between the metal
10   ion(s) and the backbone of the synthetic monooxygenase said synthetic monooxygenase being prepared by:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein which collectively
15   form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed monooxygenase apoprotein, wherein said determining comprises:

    (1) representing the structure of the host protein as a three-dimensional atomic
20   coordinate array;

    (2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

    (3) identifying volume elements in the host protein excluding the backbone of the
25   protein equal to between about 90% and 110% of the volume of the metal binding site;

    (4) combinatorially replacing sequence residues in said volume elements, thereby generating a set of primary sequence mutations;

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(5) calculating the volume change resulting from each set of primary sequence mutations;

(6) selecting set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed monooxygenase apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed monooxygenase apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed monooxygenase apoprotein;

(E) overexpressing the designed monooxygenase apoprotein in the host organism; and

(F) either (i) purifying the overexpressed designed monooxygenase apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional monoxygenase, wherein said reconstituting optionally comprises partially denaturing the designed monooxygenase apoprotein, treating the partially denatured designed monooxygenase apoprotein with the metal ion(s) or metal cluster and concurrently refolding the monooxygenase; or

(ii) incubating the metal ion(s) or metal cluster with the designed superoxide dismutase apoprotein and purifying directly an intact functional monooxygenase.

In a certain embodiment, the synthetic monooxidase is made as disclosed above, wherein the determining step comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond

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angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting synthetic monooxidase containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing

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the designed synthetic monooxidase apoprotein.

In another embodiment, the invention provides a synthetic monooxidase prepared as disclosed above wherein the monooxidase contains one metal ion binding site. An example is one containing a dioxo-Mo binding site. However, other synthetic

5   monooxygenases containing more than one metal ion binding site are within the scope of the present invention.

Preparation of the synthetic monooxidase requires initial consideration of primary sequence mutations which are determined by molecular modeling. Any of a variety of modeling protocols are available to assist in deteriming the particular primary sequence

10   mutations. Thus, primary sequence mutations are determined using an algorithm selected from the group consisting of DEZYMER, PROPAK and METALSEARCH. However, a preferred protocol for modeling uses the DEZYMER algorithm. Coordinate data are needed to apply a modeling protocol to determine the primary sequence mutations. Structure data of the host protein for use in the design of the synthetic monooxidase is

15   obtained as a three-dimensional atomic coordinate array either from X-ray crystallography, electron diffraction or neutron diffraction, if crystals are available, or from nuclear magnetic resonance spectroscopy, if no crystals are available.

Suitable host proteins useful in the construction of the synthetic monooxidase include, but are not limited to, thioredoxin or human serum albumin.

20

The present invention further provides a method of oxidizing an alkane which comprises treating the alkane with a functional metalloprotein containing at least one metal binding site wherein the metal binding site is selected from a $Mo(=O)_2$ complex and a mononuclear iron center together with a cubic iron-sulfur cluster under conditions suitable

25   to form an oxidized alkane. In one embodiment of the invention, the functional metalloprotein is selected from the group consisting of ferredoxin, human serum albumin, a catalytic antibody, a tyrosinase and a superoxide dismutase.

The present invention also provides a method of treating a subject suffering from ischemia which comprises administering to the subject a functional metalloprotein prepared

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from a host protein containing a metal binding site wherein the protein has superoxide dismutase activity in a therapeutically effective amount. In this method, the functional metalloprotein may be prepared from a wide range of host proteins, oligopeptides or polypeptides compatible with a medicinal or clinical application. A preferred host protein

5   is a human serum albumin. In one embodiment of the method, the metal ion binding site comprises Fe(II/III) and a histidine ligand.

The present invention also provides a method of treating a subject suffering from reperfusion type tissue damage which comprises administering to the subject a functional metalloprotein prepared from a host protein containing a metal binding site wherein the

10 protein has superoxide dismutase activity in a therapeutically effective amount. The host protein may be any protein, oligopeptide or polypeptide suited to medicinal application. In one embodiment, the host protein is a human serum albumin. The metal ion binding site may comprise any metal ion compatible with a medicinal application, but favorably comprises Fe(II/III) and a histidine ligand.

15 As applied in the present invention, the rational protein design algorithm DEZYMER (Hellinga, H. W. & Richards, F. M. (1991) *J. Mol. Biol.* **222**, 763-786) is used to search the three-dimensional structure of the host protein (*e.g.*, Trx) to identify locations where it is predicted to be geometrically possible to introduce mutations to form the correct primary coordination sphere. Mutations are chosen to satisfy both the intended

20 metal binding geometry, and the steric requirements of the protein fold. Once a coordination sphere has been positioned, further mutations may be introduced to retain steric compatibility between the metal binding site and the surrounding protein. All the substitutions are therefore structurally conservative in nature, the intent being to maintain the original fold and stability of the host protein.

25 The DEZYMER algorithm makes predictions based on strict geometric principles without explicit consideration of binding thermodynamics or protein dynamics. The rational design approach used here is based on the placement of an active site into the framework of a known protein fold. Active sites are described as geometrical arrangements of functionally important amino acids around a ligand, for example, a

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transition metal center. In the first phase of the process, the DEZYMER algorithm systematically examines a protein structure to identify backbone positions that are arranged in such a way that appropriate rotamers of the residues in the binding site definition can be placed to satisfy the desired ligand geometry. In the second phase, additional changes

5   may be introduced to ensure steric complementarity of the placed site with the surrounding protein matrix. This approach therefore requires two conditions: first, that the proposed mutations retain the original protein fold by maintaining sterically reasonable packing interactions (Richards, F. M. & Lim, W. A. (1993) in *An Analysis of packing in the protein folding problem, Quarterly Reviews of Biophysics,* 26, 423-98); and, second, that

10   correct geometrical presentation of ligands is a sufficiently powerful approximation to reliably predict the construction of binding sites.

It is within the scope of the present invention to design particular synthetic metalloproteins in which cuboidal $\{Fe_4S_4\}$ clusters or mononuclear metal ion sites are installed which have desriable metallopotentials, specifically adapted for a particular

15   purpose or application. proteins, which contain structurally equivalent metal clusters, are grouped in two classes, the ferredoxins (Fds) and the high potential iron-sulfur proteins (HiPIPs).

In accord with the method of the present invention, the synthetc metalloprotein comprising human serum albumin in which an active site having superoxide dismutase

20   activity, herein referred to as HSA-SOD, and related compositions are useful in treating subjects suffering from ischemia or reperfusion tissue damage. The ability of these synthetic metalloproteins to remediate symptoms of ischemia or reperfusion damage in tissue culture and in clinical subjects is supported by the effectiveness for known superoxide dismutase compositions. For example, it is known that SOD may be applied

25   to treat ischemia and reperfusion injury in various tissues such as brain (Matsumiya *et al.,* (1991) *Stroke,* 22, 1193-1200; Kirsch *et al.* (1993) *Pediatr. Res.,* 34, 530-537); spinal cord (Cuevas *et al.,* (1989) *Anat. Embryol. (Berl.),* 179, 251-255); liver (Kondo *et al.,* (1996) *J. Surg. Res.,* 60, 36-40); pancreas (Closa *et al.,* (1993) *Inflammation,* 17, 563-571); bowel (Rhee *et al.,* (1991) *Am. Surg.,* 57, 747-750); and kidney (Mihara *et al.,*

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(1994) *J. Drug Target.*, **2**, 317-321).   Other indications for which synthetic metalloproteins such as HSA-SOD have utility include trauma (head injury, multiple organ failure, subarachnoid hemorrhage), transplantation (rejection of transplant, necrosis), radiation therapy (protection of bone marrow cells, radiation-induced fibrosis and

5   cystosis), and acute infection and inflammation (burn wound healing, esophagitis, thrombosis, and influenza virus infection). Mammals, and specifically humans, suffering from ischemia or reperfusion tissue damage can be treated by administering to the subject an effective amount of an HSA-SOD or a related metalloprotein in the presence of a pharmaceutically acceptable carrier or diluent.

10   The magnitude of the therapeutic dose of the particular metalloprotein will vary with the nature and severity of the condition to be treated and with the particular metalloprotein and its route of administration. Systemic dosages to achieve relief from symptoms of ischemia and reperfusion type tissue damage typically range from 2 mg/kg to 0.0001 mg/kg per day as a single daily dose or divided daily doses. Typical dosages

15   for topical application range from 0.001 to 100% by weight of the synthetic metalloprotein.

Any suitable route of administration may be employed for providing a mammal, especially a human, with an effective dosage of a synthetic metalloprotein disclosed herein. The synthetic metalloprotein may be administered subcutaneously, intravenously,

20   intraperitoneally, intramuscularly, parenterally, orally, submucosally, by inhalation, transdermally via a slow release patch, rectally, or topically, in an effective dosage range to achieve relief from symptoms of ischemia and reperfusion type tissue damage. Dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, lotions, gels, tinctures, sprays, powders, pastes, slow-release

25   transdermal patches, suppositories for application to rectal, vaginal, nasal or oral mucosa. Thickening agents, emollients, and stabilizers can be used to prepare compositions for administration. Examples of thickening agents include petrolatum, beeswax, xanthan gum, or polyethylene, humectants such as sorbitol, emollients such as mineral oil, lanolin and its derivatives, or squalene.

All patents, applications, articles, publications and test methods mentioned above are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

The present invention will be better understood from the Materials and Methods which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described in the claims which follow thereafter.

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Materials and Methods

EXAMPLE 1

Molecular Simulations. The DEZYMER algorithm (Hellinga, H. W. & Richards, F. M. (1991) *J. Mol. Biol.* **222**, 763-85) was used to perform the calculations for the site
5   design and refinement on a NeXT computer.

EXAMPLE 2

Cloning and Protein Expression. Trx-SOD was constructed by standard PCR mutagenesis techniques using *Pfu* DNA polymerase (Stratagene) and the modified gene
10   was inserted into the expression vector pKK-T7E downstream of a T7 promoter and sequenced in full. *Escherichia coli* (HMS174) cells carrying pTrx-SOD were grown in 2xYT media supplemented with 4 ml/liter glycerol to A600 = 2.0 and induced with 0.4 mM isopropyl-β-D-thiogalactoside; the cells were harvested 4 hours post induction.

15   ## EXAMPLE 3

Purification of Apoprotein (apo) Trx-SOD. The harvested cells from 10 liters of culture were resuspended in 100 ml 20 mM Tris-Cl, pH 8, 100 mM NaCl, 5 mM EDTA and lysed by sonication. The cleared lysate was then brought to 45% saturation with solid ammonium sulfate. After centrifugation, the pellet was redissolved in a minimum volume
20   of 20 mM Tris-Cl, pH 8, 25 mM NaCl, adjusted to 50 mM 2-mercaptoethanol and 1 mM each o-phenanthroline, TIRON (4,5-dihydroxy-*m*-benzenedisulfonic acid) and EDTA to remove endogenous metals and dialyzed overnight against 20 mM Tris-Cl, pH 8, 25 mM NaCl.

The sample was then loaded onto a 200 ml DEAE-cellulose column, washed with
25   one column volume of 20 mM Tris-Cl, pH 8, 25 mM NaCl and eluted with a 25-500 mM salt gradient in 20 mM Tris-Cl, pH 8. Peak fractions were further purified by Superdex 200 column chromatography in 20 mM HEPES-Cl, pH 8, 25 mM NaCl. This purification procedure resulted in the isolation of approximately 1.2 grams of Trx-SOD of > 99% purity from a single 10 liter fermentor growth (Figure 2).

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A final purification step takes advantage of the unusual ability of the Trx host protein to thermally denature and renature reversibly; aliquots of purified apo protein were heated to 90 $^{\circ}$C for 20 min., spun in a microcentrifuge to remove any precipitated material and rechromatographed on a Superdex S75 column in 20 mM HEPES-Cl, pH 8, 25 mM

5   NaCl where only the properly folded Trx-SOD is isolated.

EXAMPLE 4

Iron Reconstitution of Trx-SOD. Reconstitution was readily accomplished by the addition of 1 equivalent of ferrous ammonium sulfate from a concentrated (100 mM)

10   aqueous stock solution prepared immediately before use. Development of a yellow chromophore, indicative of iron oxidation, was complete in minutes. The same reconstitution procedure was followed for both normal and heat-treated enzymes. Iron to protein ratios were determined by atomic absorption (Varian SpectrAA-20) and UV/vis (Perkin-Elmer Lambda 6 spectrophotometer; $\epsilon_{M, 280nm}$ = 13,700 based on total amino

15   acid analysis) spectroscopy.

Reconstitution also performed under anaerobic conditions to assess the level of iron binding. Iron binding stoichiometry was measured by varying the initial ferrous ammonium sulfate-to-Trx-SOD ratio (0.1 to 10). After extended incubation (5 hours) in 20 mM Hepes, pH 8.0, 25 mM NaCl, 21 $^{\circ}$C under strict anaerobic conditions in an inert

20   atmosphere (argon) box, the reconstitution samples were dialyzed overnight against 20 mM Tris-Cl, pH 8, 25 mM NaCl (no EDTA). No oxidation of the iron was observed as judged by the absence of any visible chromophore. After air oxidation (1 hour), Fe to Trx-SOD ratios were measured by atomic absorption and UV spectrophotometric methods; oxidation, however, was complete in minutes as judged by color development.

25

EXAMPLE 5

Activity Measurements. The superoxide dismutase activity of holo Trx-SOD was determined by a standard indirect colorimetric assay that measures the level of inhibition of superoxide induced reduction of colorless nitro blue tetrazolium (NBT, Sigma) dye to

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its oxidized blue formazan form (Flohe, L. & Otting, F. (1984) *Methods Enzymol.* 105, 93-104). One unit of SOD activity is defined as that amount of enzyme that inhibits the rate of NBT reduction, under the specified conditions, by 50%. Several dilutions of one enzyme solution were investigated in order to accurately extrapolate to the 50% inhibition

5   level. All assay reactions were performed in triplicate in 50 mM 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), pH 9.0, 0.5 mM xanthine, 0.5 mM EDTA, 40 mg/ml nitroblue tetrazolium, 21 $^\circ$C. Generation of the superoxide anion was accomplished enzymatically by the reaction of xanthine and xanthine oxidase (Flohe, L. & Otting, F. (1984) *Methods Enzymol.* 105, 93-104). Xanthine oxidase was titrated in the

10   standard cytochrome c assay (Flohe, L. & Otting, F., *supra*) to determine the amount of enzyme require to give a $\Delta Abs_{550nm} = 0.025/min.$; this amount of xanthine oxidase was then used in the NBT assays. Parallel studies were also performed with *E. coli* iron SOD obtained from commercial sources (Sigma).

15

EXAMPLE 6

Rational Design. The iron SOD site definition, derived from the X-ray crystal structures of iron SOD from *E. coli* (Stallings, W. C., *et al.,* (1983) *Proc. Nat'l Acad. Sci. USA* 80, 3884-8) and *Pseudomonas ovalis* (Stoddard, B. L., *et al.,* (1990)

20   *Biochemistry* 29, 8885-93), consisted of a trigonal bipyramidal iron center with axial histidine and exogenous ligand binding sites (for superoxide or other anions) and equatorial (N-His)$_2$(O-Asp) ligands. A bound superoxide ligand in the axial position was added to the site definition, based upon the idealized geometry of a Fe-O$_2^-$ adduct (Hill, A. A. O. & Tew, D. G. (1987) in *Dioxygen, Superoxide and Peroxide as Ligands*, eds.

25   Wilkinson, G., Gillard, R. D. & McCleverty, J. A. (Pergamon Press, Oxford), Vol. 2, pp. 315-333). The DEZYMER algorithm generated six solutions for the Fe SOD active site definition; the present inventors constructed and characterized one solution (Trx-SOD): Leu7His, Phe27Asp, Ile60His, Asn63His (Figure 1). This site was initially selected due to the relatively short axial distance between the iron center and the protein surface ($\approx$

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9Å), the favorable orientation of the "open" coordination site which is aligned towards the protein surface and not directed towards the protein interior, and the lack of any protein main chain atoms sterically hindering access to the iron site.

Trx, a relatively small (108 amino acid residues) monomeric protein that contains
5 a single redox active disulfide bond (Cys32, Cys35) (Holmgren, A. (1985) in *Ann. Rev. Biochem.* **54**, 237-271), was chosen (Hellinga, H. W., Caradonna, J. P. & Richards, F. M. (1991) *J. Mol. Biol.* **222**, 787-803) as a scaffold protein since the gene for Trx has been cloned, sequenced, and expressed at high levels (Wallace, B. J. & Kushner, S. R. (1984) *Gene* **32**, 399-401). Trx is a stable protein (oxidized Trx T_m: 86 $^\circ$C, reduced Trx T_m: 75
10 $^\circ$C) (Holmgren, A. (1985) in *Ann. Rev. Biochem.* **54**, 237-271) that accommodates conservative as well as some non-conservative mutations (Wynn, R. & Richards, F. M. (1993) *Protein Sci.* **2**, 395-403). In addition to the availability of a high-resolution (1.68 Å) X-ray structure (Katti, S. K., LeMaster, D. M. & Eklund, H. (1990) *J. Biol. Chem.* **212**, 167-184), there are several solution structures (oxidized and reduced) derived from
15 NMR spectroscopic methods (Dyson, H. J., Holmgren, A. & Wright, P. E. (1989) *Biochemistry* **28**, 7074-7078); Dyson, H. J., Gippert, G. P., Case, D. A., Holmgren, A. & Wright, P. E. (1990) *Biochemistry* **29**, 4129-4136), indicating the feasibility of obtaining structural information on the designed protein in the absence of crystals.

Several other mutations were introduced into Trx-SOD: His6Asn, to prevent
20 competition between the designed His7 site and the naturally occurring His6 residue, the only histidine residue in host thioredoxin; Leu58Ala, Thr66Ala to improve packing around the designed site owing to differences in volume between the naturally occurring residues of Trx and those of the designed Fe site; and Asp26Ala to improve the global stability of the protein (the Asp26Ala mutant of thioredoxin is 3 kcal stabilized as compared to wild-
25 type) (Langsetmo, K., *et al.* (1991) *Biochemistry* **30**, 7603-7609; Langsetmo, K., *et al.* (1991) *Biochemistry* **30**, 7609-7614). The present inventors also used the well-characterized Cys32Ser, Cys35Ser mutant of Trx to eliminate the native disulfide bond thereby precluding the possibility of sulfur coordination to the metal center and to facilitate maintenance of monomeric Trx.

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EXAMPLE 7

Iron Reconstitution of Purified Trx-SOD. The optical spectra of apo and $\{Fe^{3+}\}$Trx-SOD are presented in Figure 3. The apo protein sample has virtually no absorbance at wavelengths longer than 320 nm. The addition of one equivalent of Fe^{2+}

5   leads to the rapid incorporation and oxidation of iron to produce the 1:1 holo $\{Fe^{3+}\}$Trx-SOD protein. Immediately evident is the broad absorption maximum centered at approximately 350 nm with a long absorption tail extending beyond 500 nm. This feature, which is not observed for either apo or $\{Fe^{2+}\}$Trx-SOD, is assigned as a histidine-to-Fe^{3+} charge-transfer transition and can only arise from Fe^{3+} binding to the designed histidine-

10   rich site. The iron is tightly bound ($K_d < 1$ µM as determined by titration of Fe^{2+} assayed by fluorescence, data not shown) and cannot be removed from the protein even by extended incubation in the presence of 50 mM EDTA, suggesting that it is buried in the interior of the protein. Treatment of chemically or heat denatured $\{Fe^{3+}\}$Trx-SOD with EDTA, however, results in the formation of Fe^{3+}EDTA. The Fe^{2+} center of reduced

15   $\{Fe^{2+}\}$Trx-SOD is readily removed by addition of excess *o*-phenanthroline, a neutral iron chelator.

Fe^{3+} (d^5) has relatively slow exchange kinetics and thus it is possible, if oxidation is rapid relative to complete reconstitution, that reconstitution in air does not yield the thermodynamically favored metal binding site. To address this issue, extended incubation

20   of apo Trx-SOD with as many as 10 equivalents of Fe^{2+} followed by overnight dialysis against gel filtration buffer (no EDTA present) was carried out under strictly anaerobic conditions. Despite the excess of metal used, only one equivalent of iron is bound after dialysis, indicating that there is only one high affinity iron binding site in this protein. Furthermore, the sample develops the characteristic His-to Fe^{3+} LMCT bands in the

25   optical spectrum immediately upon exposure to air, whereas stock solutions of ferrous ammonium sulfate oxidize only slowly. The final ϵ_{M350} values of samples reconstituted aerobically and anaerobically are virtually identical ($< 5\%$ discrepancy). These results indicate that iron oxidation occurs only after incorporation into the histidine rich site and that the thermodynamically favored iron site (Fe^{2+}/anaerobic conditions) is the same as

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that found when stoichiometric reconstitution is performed in the presence of dioxygen.

The addition of exogenous anionic ligands such as N_3^- or F^- to $\{Fe^{3+}\}Trx$-SOD results in immediate spectral changes (Figures 4A and B, respectively) suggesting that these ligands are able to enter the primary coordination sphere of the iron center and form

5   coordination complexes. Azide binding induces a shift in the LMCT envelope to lower energy with the band now observed at 400 nm, while fluoride binding results in a reduction of the intensity of the 350 nm absorption and in an apparent hypsochromic shift in the LMCT envelope to higher energy with the charge transfer transition now found at 330 nm. Both the magnitude and direction of these shifts are analogous to those reported for *E.*

10   *coli* iron SOD (Slykhouse, T. O. & Fee, J. A. (1976) *J. Biol. Chem.* **251**, 5472-7). These data provide compelling evidence that the electronic structure of the designed iron site resembles *E. coli* iron SOD, has an open coordination position necessary for anion binding, and is accessible to endogenous anions.

15   <div align="center">**EXAMPLE 8**</div>

Catalytic Dismutase Activity. SODs are usually assayed indirectly owing to the extreme instability of the superoxide ion in aqueous solution. A common assay method utilizes xanthine oxidase and xanthine to generate the superoxide ion which then reduces nitroblue tetrazolium (NBT), a reaction which can be followed spectrophotometrically;

20   inhibition of this reduction is a measure of SOD activity (Flohe, L. & Otting, F. (1984) *Methods Enzymol.* **105**, 93-104). The NBT assay is extremely reproducible provided there is sufficient total protein present in the assay to keep the formazan product of NBT reduction soluble. The results of the SOD assay for heat treated $\{Fe^{3+}\}Trx$-SOD at a single fixed pH (9.0) are given in Figure 5. $\{Fe^{3+}\}Trx$-SOD inhibits the reduction of NBT

25   in proportion to the amount of enzyme added over a large concentration range, indicating that the designed protein does indeed show superoxide dismutase activity.

To address the relative rates of catalysis between the native and designed enzymes, samples of *E. coli* Fe SOD were assayed in parallel with $\{Fe^{3+}\}Trx$-SOD. This direct comparison indicated that the designed enzyme catalyzes the dismutation reaction at a rate

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$\approx 10^4$-fold slower than native Fe SOD, demonstrating that $\{Fe^{3+}\}$Trx-SOD is a highly active protein able to catalyze the dismutation reaction with $k \approx 10^5$ $M^{-1}s^{-1}$. Apo Trx-SOD does not show any activity in this assay, nor does $\{Fe^{3+}\}$Trx-SOD that has been boiled for 5 minutes and centrifuged immediately prior to assay. To control against the

5   possibility that free metal ions in solution could contribute to the observed activity, EDTA was included in all assay buffers. Furthermore, Fe^{3+}-EDTA demonstrated no activity when assayed, in agreement with previous reports (Bull, C., *et al.* (1982) *Arch. Biochem. Biophys.* **215**, 551-5).

As native *E. coli* Fe SOD catalyzes superoxide dismutation at a rate on the order

10   of 10^9 $M^{-1}s^{-1}$ (Bannister, *et al.* (1987) *CRC Crit. Rev. Biochem.* **22**, 111-80), it is essential to demonstrate that the observed enzymatic activity of $\{Fe^{3+}\}$Trx-SOD cannot be attributed to trace contamination with *E. coli* SOD. Several lines of evidence strongly contradict this possibility. First, as discussed above, the present preparations of apo Trx-SOD do not show any dismutase activity as isolated. Second, the activity of *E. coli* Fe

15   SOD is reduced at least 1000-fold (lower limit) by the heat-treatment step. Native *E. coli* Fe SOD was heated to 90 °C for 10 minutes., spun to remove the large precipitate, and then assayed for activity. No dismutase activity was observed in the heat-treated native *E. coli* enzyme, even when assayed at 1000 times the normal enzyme concentration. Reconstitution of apo Trx-SOD by our standard protocol either before or after heat-

20   treatment results in enzymes with little or no difference in dismutase activity, indicating that the observed activity cannot arise from contamination by the heat-labile native *E. coli* enzyme. Furthermore, treatment of *E. coli* Fe SOD with reductant and chelators (a routine part of the purification procedure as described in Materials and Methods) yields an enzyme that still precipitates upon heat-treatment (and is thus easily removed), ruling

25   out the possibility that the apo form of the *E. coli* dismutase enzyme might be carried along in a soluble form that can be reconstituted in parallel with Trx-SOD.

A third argument countering the possibility of trace contamination of samples of the synthetic metalloprotein of the present invention takes into account the high degree of purity of the presently disclosed Trx-SOD preparations (Fig. 2). The activity of Trx-SOD

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is about 10^{-4} times that of native *E. coli* Fe SOD; that is, the *E. coli* enzyme would need to be present at levels of 0.01% to account for the observed activity. However, since it has been demonstrated that heat-treatment of *E. coli* Fe SOD reduces its activity by *at least* 1000-fold (a lower limit), the contaminant would have to comprise 10% of present
5   preparations to account for the observed activity after heat-treatment. Examination of Fig. 2 excludes this possibility.

Finally, iron-limiting reconstitution experiments provides positive evidence that it is the iron in the designed site that is catalytically active. Samples of apo heat-treated Trx-SOD (>99% pure; Figure 2) were reconstituted to Fe:protein ratios of 0, 0.25, 0.50, 0.75
10   and 0.95 and assayed for activity (Figure 6). Trx-SOD shows no activity when iron is absent and dismutase activity increases in proportion to the degree of iron incorporation (Figure 6). Again, these data indicate that the observed activity is associated with the designed protein and is not due to trace amounts of native *E. coli* Fe SOD. Furthermore, the parallel between activity and the intensity of the His-to-Fe^{3+} LMCT band indicates that
15   it is iron in the designed histidine rich site that is catalytically active.

EXAMPLE 9

Cloning and Protein Expression. Trx-Fe_4S_4 was constructed by Kunkel mutagenesis techniques and the modified gene was inserted into the expression vector
20   pKK-T7E (Kriwacki, R. W., *et al.*, (1992) *Proc. Nat'l Acad. Sci. USA* **89**, 9759-9763) downstream of a T7 promoter and sequenced in full. Expression of Trx-Fe_4S_4 was achieved in *E. coli* BL21(DE3). pTrx-Fe_4S_4/BL21 was grown in 2xYT, 50 µg/ml Ampicillin, at 25 °C, and induced with IPTG; cells were harvested 4 hours post induction.

25

EXAMPLE 10

Purification of apo Trx-Fe_4S_4. The harvested cells from 10 liters of culture were resuspended in 140 ml 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM EDTA and lysed by sonication. The cleared lysate was brought to 0.25% PEI, centrifuged, and then

brought to 45% saturation with solid ammonium sulfate and clarified. The supernatant
was then brought to 90% saturation with solid ammonium sulfate. After centrifugation,
the pellet was dissolved in 50 ml of 50 mM Tris-Cl, pH 8.1, 20 mM NaCl, and dialyzed
against the same buffer for 4 hours. The volume was reduced by dialysis against solid

5   PEG 8000 at room temperature for 3 hours, followed by dialysis against 50 mM Tris-Cl,
pH 8.1, 20 mM NaCl for 6 hours.

The sample was then applied in approximately 60 1.2 ml portions onto a 16 mm
x 60 cm S75 gel filtration column and eluted with 50 mM Tris-CL, pH 8.1, 20 mM NaCl,
4 $^{\circ}$C. Peak fractions were then combined and loaded onto a 25 mm x 7 cm DEAE-

10   cellulose column, washed with five column volumes of 50 mM Tris-Cl, pH 8.1, 20 mM
NaCl and eluted with a 20-500 mM salt gradient in 20 mM Tris-Cl, pH 8.1. The isolated
protein was concentrated four-fold in an ultrafiltration cell and an equal volume of glycerol
was added. Solutions of purified apo Trx-Fe$_4$S$_4$ were made 100 mM in DTT prior to
storage at -70 $^{\circ}$C. This purification procedure resulted in the isolation of approximately

15   600 mg of apo Trx-Fe$_4$S$_4$ of > 99% purity from a single 10 liter fermentor growth.

EXAMPLE 11

Cluster Reconstitution of Trx-Fe$_4$S$_4$. Reconstitution of apo Trx-Fe$_4$S is
performed under strict anaerobic conditions in an inert (argon) atmosphere box using

20   rigorously deoxygenated solutions. Apo Trx-Fe$_4$S$_4$ is passed through a gel filtration
column eluted with 15 mM CHES, pH 8.5, to remove glycerol and DTT. The resulting
solution is then made 2.0 M urea and 10 mM β-mercaptoethanol (βME) and allowed to
sit for 15 minutes at 25 $^{\circ}$C. A freshly prepared solution of the synthetic cluster {Fe$_4$S$_4$(S-
EtOH)$_4$}(Me$_4$N)$_2$ Christou, G. & Carner, C. D. (1979) *Journal of the Chemistry Society,*

25   *Dalton Transactions* , 1093-1094 in dimethyl sulfoxide (DMSO) is added to the urea
treated apo Trx-Fe$_4$S$_4$ protein in a 1:1 (cluster:protein) molar ratio, keeping the DMSO
content of the protein containing solution <1.5%. After 5 minutes, the solution is diluted
with an equal volume of 15 mM CHES, pH 8.5 solution. Excess FeS, βME, and residual
urea are removed by desalting on a PD10 column equilibrated in 10 mM MOPS, pH 7.4,

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100 mM NaCl. Reconstitution occurs with > 90 % recovery of protein. Fe:S^{2-}:protein ratios are determined by atomic absorption spectroscopy (Varian SpectrAA20), colorimetric Beinert, H. (1983) *Analytical Biochemistry* **131**, 373-378 and UV (reduced apo Trx-Fe$_4$S$_4$: ϵ_M = 13 700 verified by total amino acid analysis) spectrophotometric

5   methods, respectively.

EXAMPLE 12

Measurement of in vivo activity of thioredoxin. An M13mp19 recombinant phage containing the gene for Trx-Fe$_4$S$_4$ was plated on a bacterial host strain deleted for

10   wild-type thioredoxin (A307, (23, 24)). This strain will not support growth of wild-type M13mp19 phage, but will support the growth of a M13mp19 recombinant phage into which an active thioredoxin has been cloned. Activity was scored by comparing phage titers on the Δ*trxA* (A307) strain and its parent *trxA*[+] strain, K38, of phage stock that had been grown on the permissive strain DH5αF′: a recombinant phage titer within 1 order of

15   magnitude on both strains (typically 10^{10} to 10^{11} particles/ml) was scored as displaying thioredoxin activity (inactive recombinants give titers of 10^5 to 10^6 particles/ml).

Spectroscopic Studies. UV/vis electronic spectra were recorded under strict anaerobic conditions in gas tight optical cuvettes (1 cm pathlength) with a Perkin Elmer Lambda 6 spectrometer. CD spectra were obtained with an AVIV Model 60DS Circular

20   Dichroism Spectropolarimeter using a quartz cell with a 1 mm pathlength at 25 °C at a protein concentration of approximately 25 μM. EPR spectra were collected on a Varian E-line EPR spectrometer operating at a microwave frequency of 9.24 GHz, with 1.5 mW microwave power, 10 G modulation amplitude at 25 K.

25

DISCUSSION

In accord with the present invention, the rational design algorithm, DEZYMER,

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is used to introduce a catalytically active iron SOD site into the hydrophobic core of *E. coli* thioredoxin (Trx), a protein normally devoid of transition metal centers. Inspection of the designed site reveals several favorable properties. Thioredoxin folds to form a hydrophobic core of β-pleated sheet flanked on either side by two α-helices; the structure

5   can be considered as being formed of two domains, βαβαβ from residues 1 to 59 and ββα from residues 76 to 108 (Katti, S. K., LeMaster, D. M. & Eklund, H. (1990) *J. Biol. Chem.* **212**, 167-184). Two of the designed coordinating residues (His7, Asp27) lie on the β-sheet, the other resides (His60, His63) on an α-helix. The iron coordination sphere is encapsulated by a hydrophobic shell (Phe12, Val16, Val25, Ala58, Ala66, Ala67, Ile72,

10   Leu79), a feature found in native Fe SODs and thought to be necessary for activity (Stoddard, B. L., *et al.*, (1990) *Biochemistry* **29**, 8885-93). Finally, the designed axial superoxide coordination position, though buried, is only 7-9 Å from the surface, with no intervening main chain atoms. It is also possible to modify residues near the iron active site that would allow hydrogen bonding to a bound superoxide ion in further rounds of

15   mutagenesis. Thus this design contains many of the structural features thought to be necessary for the dismutase reaction.

Three primary chemical features must be realized if the designed iron center is to catalyze the dismutation of superoxide ion (Fee, J. A. (1980) in *Superoxide, Superoxide Dismutases and Oxygen Toxicity*, ed. Spiro, T. A. (Wiley-Interscience, New York), Vol.

20   2, pp. 209-239): (I) the iron center must have a minimum of one coordination site available for binding superoxide in two adjacent oxidation states (Fe^{2+}/Fe^{3+}); (ii) the iron redox couple, governed in part by the primary coordination sphere as well as the surrounding protein matrix, must lie between the redox potentials of superoxide oxidation (0.16 V) and superoxide reduction (0.89 V); and (iii) the iron center must be able to cycle

25   between the Fe^{2+} and Fe^{3+} oxidation states more rapidly than the rate of the spontaneous dismutation reaction.

Evidence for iron incorporation into the designed site comes from optical studies of holo Trx-SOD. The electronic absorption spectra of high-spin d^5 (Fe^{3+}) centers are typically dominated by the spin-allowed ($\epsilon_M > 1000$) ligand-to-Fe^{3+} charge-transfer

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(LMCT) transitions originating from relatively easily oxidized ligands such as histidine (Zhang, Y., Gebhard, M. S. & Solomon, E. I. (1991) *J. Am. Chem. Soc.* **113**, 5162-5175, Averill, B. A. & Vincent, J. B. (1993) *Methods Enzymol.* **226**, 33-51). Electronic transitions arising from within the *d*-orbital manifold (ligand-field, LF) are all spin-

5   forbidden and are therefore much less intense ($\epsilon_M < 500$). Transitions analogous to those observed in $\{Fe^{3+}\}$Trx-SOD ($\epsilon_{M350} = 2400$) are reported for ferric SODs from bacterial and algal sources ($\epsilon_{M350} = 1675$-2860) (Averill, B. A. & Vincent, J. B. (1993) *Methods Enzymol.* **226**, 33-51) as well as for ferric soybean lipoxygenase ($\epsilon_{M350} \approx 2000$) Averill, B. A., *supra*). Based on X-ray crystallographic characterization of both Fe^{3+} SOD

10   (Stoddard, B. L., *supra*) and soybean lipoxygenase (Boyington, J. C., Gaffney, B. J. & Amzel, L. M. (1993) *Science* **260**, 1482-1486) that show the presence of three and four bound His ligands respectively, the absorption bands observed only in oxidized $\{Fe^{3+}\}$Trx-SOD are assigned as endogenous His-to-Fe^{3+} LMCT transitions, although bound carboxylate may also contribute to this region. Both our qualitative and quantitative data

15   indicate that a single Fe^{3+} ion binds tightly to a histidine rich coordination sphere, which is consistent with metal binding occurring in the designed three-histidine site. These spectroscopic techniques, however, do not allow the definitive determination of Asp27 (carboxylate) coordination.

The addition of anions to solutions of $\{Fe^{3+}\}$Trx-SOD produced changes in the

20   optical spectra that also parallel the reported behavior native Fe SOD enzymes (Averill, B. A., *supra*). A 2.9 Å resolution structure of iron superoxide dismutase from *Pseudomonas ovalis* complexed with the inhibitor azide (Stoddard, B. L., Ringe, D. & Petsko, G. A. (1990) *Protein Eng.* **4**, 113-9) supports the assignment of the origin of the optical LMCT band as arising from the anion active site complex. These data also indicate

25   the ability of small anions to readily bind to the designed active site of $\{Fe^{3+}\}$Trx-SOD, a necessary step if an inner-sphere reaction is to occur during the dismutation reaction.

The native *E. coli* Fe SOD falls into the class of "perfectly evolved" enzymes that operate at the diffusion-controlled limit (Bannister, J. V., Bannister, W. H. & Rotilio, G. (1987) *CRC Crit. Rev. Biochem.* **22**, 111-80). Studies have also shown that copper-

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manganese- and iron-containing complexes can also catalyze the dismutation of superoxide anion at this same diffusion-controlled limit (Weiss, R. H., *et al.* (1993) *J. Biol. Chem.* **268**, 23049-23054; Goldstein, S., Czapski, G. & Meyerstein, D. (1990) *J. Am. Chem. Soc.* **112**, 6489-6492; Allen, A. O. & Bielski, B. H. J. (1982) in *Formation and Disappearance of Superoxide Radicals in Aqueous Solution*, ed. Oberly, L. W. (CRC Press, Boca Raton, Fl), Vol. 1, pp. 125-142). Therefore, the chemical basis for the difference in activity between the designed enzyme and either the native SOD enzymes or small molecule model systems is of interest. Prior studies on native bacterial Fe SOD showed that the maximal turnover number is independent of pH over the range 7.5 - 10.5 (Bull, C., *et al.*, (1982) *Arch. Biochem. Biophys.* **215**, 551-5). However, K_m is dramatically influenced by pH; steady state kinetic studies show the presence of an essential ionizable group close to the iron center in the reduced enzyme with pK_a near 9.0, whose acid form appears to promote binding of superoxide to the reduced protein. Kinetic studies of iron SOD in D_2O and H_2O report a strong dependence of k_{cat} on the solvent isotope present, suggesting that the rate limiting step of catalysis is proton transfer from solvent H_2O to an ionizable active-site residue (Bannister, J. V., *supra*). The direct protonation of bound superoxide anion with solvent H_2O is unlikely in view of paramagnetic NMR studies which indicate that water exchange with the active site is too slow to be mechanistically important (Villafranca, J. J., Yost, F., Jr. & Fridovich, I. (1974) *J. Biol. Chem.* **249**, 3532-6). Crystallographic analyses of azide-inhibited Fe SOD are consistent with this interpretation and have implicated conserved tyrosine (Tyr34) and glutamine (Gln70) residues as playing possible roles in mediating protonation of superoxide through exchange with solvent (Stoddard, B. L., *supra*) The presently disclosed Trx-SOD enzyme model lacks these conserved secondary active site residues, suggesting that alternative pathways for proton transfer may be operating at slower rates, thereby decreasing the overall rate of superoxide dismutation.

Unfavorable electrostatic interactions between the Trx-SOD protein surface and the incoming anion may also play a role in diminishing the activity of $\{Fe^{3+}\}$Trx-SOD as compared to *E. coli* FeSOD. Favorable electrostatic attraction of the negatively-charged superoxide ion is known to play a role in rapid rate of dismutation by native SODs

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(Bannister, J. V., *supra*; Getzoff, E. D., *et al.*, (1992) *Nature* **358**, 347-351).   A comparison of the surface electrostatic potential of *E. coli* FeSOD and Trx-SOD reveals that the native enzyme has a strong overall positive charge on the surface near the superoxide coordination position, but that the comparable region in Trx-SOD has a high

5   concentration of negative charge.   Thus, mutation of surface residues to enhance electrostatic attraction of the negative superoxide ion is expected to boost the overall rate of reaction.   Moreover, the designed enzyme is still highly active, with superoxide dismutation rates on the order of $10^5 \, M^{-1} \, s^{-1}$.

The activity of Trx-SOD is less than that of aqueous Cu^{2+} ion and several synthetic

10   model systems whose activities are comparable to the SOD enzyme itself (Valentine, J. S. (1994) in *Dioxygen Reactions*, eds. Bertini, I., Gray, H. B., Lippard, S. J. & Valentine, J. S. (University Science Books, Mill Valley), pp. 253-314).   However, soluble metal complexes may unpredictably show catalytic dismutase activity ranging from very high to zero, depending on details of the coordination sphere that are not completely defined

15   (Weiss, R. H., *et al.* (1993) *J. Biol. Chem.* **268**, 23049-23054; Allen, A. O. & Bielski, B. H. J. (1982) in *Formation and Disappearance of Superoxide Radicals in Aqueous Solution*, ed. Oberly, L. W. (CRC Press, Boca Raton, Fl), Vol. 1, pp. 125-142).   Although a detailed comparison of Trx-SOD and the functional small molecule SOD mimics will require the completion of both structural characterization and mechanistic/kinetic studies

20   of Trx-SOD, it is reasonable to expect that restricted access to the designed active iron site and the unfavorable electrostatic attraction beween Trx-SOD and the negatively charged superoxide anion are responsible for the lower activity of Trx-SOD versus the small molecule models.

In sum, key aspects of the present approach have been realized: (I) the present

25   inventors have successfully created a mononuclear non-heme iron binding site whose electronic spectrum closely parallels that of the native enzyme; (ii) the iron site is capable of binding anions and exhibiting those spectral features reported for anion adducts of Fe SOD; and (iii) the designed site readily catalyzes the dismutation of superoxide anion. In addition, understanding the chemical basis for the differential activity of $\{Fe^{3+}\}$Trx-SOD

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versus wild-type iron SOD will allow insights into the fundamental roles played by the protein matrix in modulating active site catalytic chemistry.

Bioinorganic chemistry has traditionally focused its efforts in two directions, the small molecule, synthetic analogue approach (Ibers, J. A. & Holm, R. H. (1980) *Science*

5 **209**, 223-235) and the study of intact metalloproteins. Small molecules permit the investigation of the effects of varying the primary coordination sphere, but cannot provide the elaborate overall environment or long range interactions created by insertion into a protein shell. Conversely, study of intact metalloproteins does not frequently provide the opportunity to explore the varied contributions of the protein matrix that are often invoked

10 in mechanistic discussions. This protein design methodology allows the incorporation of both approaches into a single line of research; intact proteins can now be manipulated to vary properties of not only the immediate coordination environment but also the adjacent protein matrix while maintaining the overall structural or physiological properties of the chosen host protein.

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EXAMPLE 12

Rational Design. The $\{Fe_4S_4Cys_4\}^{n-}$ site definition was derived from the X-ray

5   crystal structure of oxidized *C. vinosum* HiPIP (1HIP)(Carter, C. W. J., *et al.*, (1972)

Proc. Nat'l Acad. Sci. USA **69**, 3526-3529). However, since cluster structures vary little

between different proteins and core oxidation states (Berg, J. M. & Holm, R. H. (1982)

in *Iron-Sulfur Proteins*, ed. Spiro, T. G. (Wiley-Interscience, New York), Vol. 4, pp. 1-

66), a template which includes all possible cysteine rotamers to allow maximum flexibility

10   should be considered as a generalized $\{Fe_4S_4(Cys)_4\}^{n-}$ core. The DEZYMER algorithm

generated several solutions for the $\{Fe_4S_4Cys_4\}^{n-}$ site definition; the present inventors

report the construction and characterization of one solution (Trx-Fe_4S_4; Leu24Cys,

Leu42Cys, Val55Cys, and Leu99Cys, Figure 7A). In addition to the four mutations

required to introduce the $\{Fe_4S_4\}$ cluster binding residues, Cys32Ser and Cys35Ser

15   mutations were made to remove the native Trx disulfide bond, thereby eliminating any

interaction from cysteine residues that are not part of the designed site. An isosteric

Asp26Leu mutation was also introduced to improve the global stability of the protein; the

Asp26Ala mutant of thioredoxin is stabilized by 3 kcal compared to the wild-type protein

(Langsetmo, K., Fuchs, J. A. & Woodward, C. (1991) *Biochemistry* **30**, 7603-7609,

20   Langsetmo, K., Fuchs, J. A., Woodward, C. & Sharp, K. A. (1991) *Biochemistry* **30**,

7609-7614). The $\{Fe_4S_4(Cys)_4\}^{n-}$ design moiety represents an isovolume exchange; there

is only a negligible decrease in the volumes occupied by amino acid side-chains in the

interior of engineered Trx-Fe_4S_4 relative to thioredoxin (Figure 7B).

Trx, a relatively small (108 amino acid residues) monomeric protein that contains

25   a single redox active disulfide bond (Cys32, Cys35) (Holmgren, A. (1985) *Annu. Rev.*

Biochem. **54**, 237-271), was chosen (Hellinga, W. H., Caradonna, J. P. & Richards, F. M.

(1991) *J. Mol. Biol.* **222**, 787-803) as an initial scaffold protein since the gene for Trx has

been cloned, sequenced, and expressed at high levels (Wallace, B. J. & Kushner, S. R.

(1984) *Gene* **32**, 399-401). Trx is a stable protein (oxidized Trx T_m: 86 °C, reduced Trx

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T_m: 75 °C) (Holmgren, A., *supra*) that accommodates conservative as well as some non-conservative mutations (Wynn, R. & Richards, F. M. (1993) *Protein Sci.* 2, 395-403). In addition to the availability of a high-resolution (1.68 Å) X-ray structure (Katti, S. K., LeMaster, D. M. & Eklund, H. (1990) *J. Mol. Biol.* 212, 167-184), there are several

5   solution structures (oxidized and reduced) derived from NMR spectroscopic methods (Dyson, H. J., Holmgren, A. & Wright, P. E. (1989) *Biochemistry* 28, 7074-7078; Dyson, H. J., *et al.*, (1990) *Biochemistry* 29, 4129-4136), indicating the feasibility of obtaining structural information on the designed protein in the absence of crystals.

10   **EXAMPLE 13**

        Folding of Designed Protein. Since the designed substitutions are conservative
in nature, maintenance of the protein fold is one of the design aims. The presence of a
near-native Trx fold can be tested *in vivo* using the absolute requirement of Trx for phage
M13 growth (Russel, M. & Model, P. (1986) *J. Biol. Chem.* 261, 14997-15005; Hellinga,

15   W. H., Caradonna, J. P. & Richards, F. M. (1991) *J. Mol. Biol.* 222, 787-803, Russel, M.
& Model, P. (1985) *Proc. Nat'l Acad. Sci. U.S.A.* 82, 29-33). Studies have shown that
the redox properties of Trx are not necessary as the double Cys32Ser, Cys35Ser mutant
is capable of catalyzing phage assembly (Russel, M. & Model, P. (1986) *J. Biol. Chem.*
261, 14997-15005). Mutagenesis studies suggest that the region important for protein-

20   protein interactions in thioredoxin function is formed by residues Gly33, Pro34, Ile75,
Pro76, Val91, Gly92 and Ala93 (Eklund, H., *et al.*, (1984) *EMBO J.* 3, 1443-1449). It
was found that $Trx\text{-}Fe_4S_4$ supported a normal phage titer when expressed in a non-permissive ΔTrx^- *E. coli* strain, providing strong evidence that the designed protein adopts
a native Trx conformation (Eklund, H., *supra*)

25   Further evidence for the proper folding of $Trx\text{-}Fe_4S_4$ is observed in the CD
spectrum (195-270 nm) of purified apo $Trx\text{-}Fe_4S_4$ (inset, Figure 8), which is identical to
that of native Trx. In addition, both apo $Trx\text{-}Fe_4S_4$ and native Trx have identical gel
filtration retention times (FPLC 16 mm x 60 cm Superdex 75pg, Pharmacia), indicating
equivalent Stokes radii. The combined phage assay results, CD spectrum, and gel filtration

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data strongly suggest that the overall aggregate structural elements of Trx are unaltered in the designed Trx-Fe_4S_4 protein.

EXAMPLE 14

Cluster Reconstitution of Purified Trx-Fe_4S_4. As isolated, the overexpressed Trx-Fe_4S_4 contains no Fe or elemental S. Reconstitution of apo Trx-Fe S was accomplished by exploiting the cooperative and reversible chemical denaturation process of the designed host protein (the midpoint of reduced apo Trx-Fe_4S_4 unfolding is 2.7 M urea *versus* 4.6 M urea for reduced Trx) under strict anaerobic conditions in an Ar flushed glove box at 25 $^\circ$C. The fully reduced protein was partially unfolded with 2.0 M urea in the presence of 10 mM mercaptoethanol and high pH (8.5) buffer. A synthetic, preformed tetranuclear iron-sulfur cluster (Hill, C. L., *et al.*, (1977) *J. Amer. Chem. Soc.* **99**, 2549-2557, Christou, G. & Carner, C. D. (1979) *J. Chem. Soc. Dalton Trans.*, 1093-1094), {$Fe_4S_4(S-EtOH)_4$}($Me_4N)_2$, dissolved in DMSO containing free βME (10 mM) was added to the unfolded protein in a 1:1 cluster:protein stoichiometric ratio. This soluble cluster is stable (<10%/hr loss) under these reaction conditions. Cluster incorporation is driven by ligand exchange processes where the less basic cysteine thiolate readily replaces the more basic mercaptoethanol ligand (Berg, J. M. & Holm, R. H. (1982) in *Iron-Sulfur Proteins*, ed. Spiro, T. G. (Wiley-Interscience, New York), Vol. 4, pp. 1-66). Trx-Fe_4S_4 was then refolded by 1:1 dilution into the same high pH buffer, followed by removal of the remaining urea using gel filtration to exchange into 100 mM NaCl, 15 mM MOPS pH 7.4, 4 $^\circ$C. During this separation step, the cluster remained completely bound to the protein. Reconstituted Trx-Fe_4S_4 contains 3.9 ± 0.2 moles of iron (atomic absorption spectroscopy) and 3.9 ± 0.2 moles of acid labile sulfide (Beinert, H. (1983) *Anal. Biochem.* **131**, 373-378) per mole of protein (reduced apo Trx-Fe_4S_4: ϵ_M = 13 700), consistent with the incorporation of a cuboidal {Fe_4S_4}$^{n+}$ cluster. Gel filtration studies (10 mm x 35 cm Superdex 75, Pharmacia) of Trx-Fe_4S_4 show a single dominant species with the identical gel filtration retention time as wild type Trx, indicating that holo Trx-Fe_4S_4 is a monomer and retains the global structure of Trx and apo Trx-Fe_4S_4.

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EXAMPLE 15

Spectroscopic Properties of holo Trx-Fe$_4$S$_4$. The UV/vis spectrum of holo Trx-Fe$_4$S$_4$ (Figure 8) purified by gel filtration chromatography shows the broad visible absorbances ($\epsilon_{M,413}$ = 16100, based on total amino acid analysis) characteristic of

5   {Fe$_4$S$_4$(Cys)$_4$}$^{2-}$ proteins 1.   (Johnson, M. K. (1994) in *Iron-Sulfur Proteins*, ed. King, R. B. (John Wiley & Sons, Oxford), Vol. 4, pp. 1896-1915, Spiro, T. G. (1982) in *Iron-sulfur Proteins* (John Wiley & Sons, New York), Vol. 4, 423). Based on SCF-Xα-SW theoretical models of the electronic structure of {Fe$_4$S$_4$(SR)$_4$} clusters, the observed bands are proposed to arise from (RS$^-$,S^{2-})-to-Fe charge transfer excitations (Yang, C.Y.,

10   *et al.*, (1975) *J. Amer. Chem. Soc.* **97**, 6596-6598). These studies suggest that the lower energy bands (420 nm), which show the most pronounced shifts ($\Delta\lambda_{max}$ ≈ 40 nm), involve excitation from orbitals with primarily S^{2-} lone pair character, as these sites are less shielded from environmental effects than the thiolate sulfur centers. The smaller shift of the higher energy transition at 315 nm ($\Delta\lambda_{max}$ ≈ 12 nm) is thought to reflect differences

15   in the basicity of the terminal mercaptoethanol and Cys thiolate ligands.

Figure 8 also shows that the predicted perturbation to the electronic spectrum of the iron-sulfur cluster after its inclusion in the interior of the designed protein relative to the spectrum of the free synthetic cuboidal cluster is observed (Hill, C. L., *et al.*, (1977) *J. Amer. Chem. Soc.* **99**, 2549-2557). A pronounced bathochromic shift of the iron-sulfur

20   chromophore upon changing solvents from H$_2$O to DMSO has been observed for both analogue clusters and iron-sulfur proteins and is believed to arise from a decrease in cluster solvent accessibility (Hill, C. L., *supra*). This behavior is analogous to the shielding of a cluster initially in aqueous medium by a lower dielectric medium as would be expected for reconstitution of Trx-Fe$_4$S$_4$.

25   The stability of the protein-based chromophore (< 5% decomposition hr^{-1}, 4°C, Ar atmosphere) is in direct contrast to the stability of the free synthetic cluster, which readily decomposes ($t_{1/2}$ ≈ 3 min.) in the absence of high concentrations of stabilizing exogenous thiol ligands (Figure 9). These observations indicate that an intact cluster is sequestered in the interior of holo Trx-Fe$_4$S$_4$, thereby shielding it from hydrolytic cluster

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degradation pathways. These optical data of Trx-Fe_4S_4 are therefore consistent with a buried {Fe_4S_4} cuboidal cluster.

As isolated, reconstituted Trx-Fe_4S_4 is EPR silent, with only minor contributions (0.08 spins/protein) believed to arise from paramagnetic cluster contamination originating from {Fe_4S_4} cluster fragmentation. This resting state property is a direct consequence of the incorporation of a synthetic cluster consisting of {$Fe^{2+}Fe^{3+}$}$_2$ in which one {$Fe^{2+}Fe^{3+}$} (S = 9/2) unit is antiferromagnetically coupled to a second {$Fe^{2+}Fe^{3+}$} (S = 9/2) to yield a S = 0 cluster (Figure 10) (Berg, J. M. & Holm, R. H. (1982) in *Iron-Sulfur Proteins*, ed. Spiro, T. G. (Wiley-Interscience, New York), Vol. 4, pp. 1-66). Upon oxidation of holo Trx-Fe_4S_4 with potassium ferricyanide (1.0 mM final concentration, *ca.* 10 equivalents), an EPR-active species is formed whose spectral features are analogous to the {$Fe_4S_4(Cys)_4$}$^-$ clusters found in oxidized HiPIPs. This species, which is observed over a wide temperature range (5K to >40K), consists of a dominant low field absorptive signal at g= 2.06, a major derivative signal at g = 2.03, and a high field signal at g=2.01 ($g_{avg} \approx 2.03$). The low intensity signals at g = 2.15, 2.10 and 1.97, not observed in the diamagnetic Trx-Fe_4S_4 resting state, originate from the ferricyanide oxidation of the iron-sulfur protein. The major and minor groups of EPR features saturate together with $P_{1/2}$ = 11.8 mW at 25 K and are indicative of transition metal-based unpaired electron density; signals typically integrate to 0.4 spins/protein.

The EPR properties of Trx-Fe_4S_4 are analogous to those reported for HiPIPs in that both are EPR active only upon oxidation, display signals with $g_{avg} > 2$, and exhibit similar microwave power and temperature dependencies (Johnson, M. K. (1994) in *Iron-Sulfur Proteins*, ed. King, R. B. (John Wiley & Sons, Oxford), Vol. 4, pp. 1896-1915). Although the observed g values of 2.06, 2.03 and 2.01 vary from those g values reported for natural HiPIP centers (Bryson, J. W., *et al.*, (1995) *Science* 270, 935-941), the present inventors consider that this may be a consequence of the protein environment that surrounds the cluster or to subtle structural distortions of the {Fe_4S_4} cluster itself imposed by the designed protein host. Studies examining the Mössbauer and MCD properties of holo Trx-Fe_4S_4 would shed light on the electronic coupling within the

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sequestered cluster. Attempts to generate the reduced paramagnetic $\{Fe_4S_4(Cys)_4\}^{3-}$ cluster with dithionite (≤ 100 equiv.) lead only to cluster degradation. This oxidation/reduction behavior is a characteristic of HiPIP iron-sulfur centers, and distinguishes them from ferredoxins and from other cluster types (Orme-Johnson, W. H.

5   & Orme-Johnson, N. R. (1982) in *Iron-Sulfur Proteins: The Problem of Determining Cluster Type*, ed. Spiro, T. G. (Wiley-Interscience, New York), Vol. 4, pp. 67-96).

While HiPIP $\{Fe_4S_4(Cys)_4\}^-$ clusters are typically characterized by axial EPR signals with $g_{avg} > 2$, reduced ferredoxin $\{Fe_4S_4(Cys)_4\}^{3-}$ clusters typically show rhombic EPR signals ($g = 2.06, 1.92, 1.88$) with $g_{avg} < 2$ (1, 39). The EPR data in Figure 10

10   similarly rule out a dominant $\{Fe_3S_4\}$ cluster, whose oxidized ground state should contain three antiferromagnetically Fe^{3+} ($S = 5/2$) centers yielding an $S = \frac{1}{2}$ ground state characterized by isotropic or axial EPR signals exhibiting g_{avg} values between 2.02 - 1.96 with a low-field g value of 2.02 (Johnson, M. K. (1994) in *Iron-Sulfur Proteins*, ed. King, R. B. (John Wiley & Sons, Oxford), Vol. 4, pp. 1896-1915). As solvent accessible

15   $\{Fe_4S_4(Cys)_4\}^{2-}$ and $\{Fe_4S_4(S\text{-}EtOH)_4\}^{2-}$ clusters are stable to reduction but not oxidation, and exhibit EPR signals with $g_{avg} < 2$ (R'zaigui, M., *et al.* (1980) *Biochem. Biophys. Res. Comm.*, **92**, 1258-1265), the spectral properties of Trx-Fe_4S_4 can not be due to a cluster bound to the exterior of the protein, in agreement with the interpretation of the optical spectrum data.

20

DISCUSSION

The present inventors have used the rational design algorithm, DEZYMER, to introduce a cuboidal $\{Fe_4S_4\}$ cluster into the hydrophobic interior of *E. coli* thioredoxin

25   (Trx), a protein normally devoid of transition metal centers. Inspection of the designed site reveals several favorable properties. Thioredoxin folds to form a core of β-pleated sheet flanked on either side by two α-helices; the structure can be considered as being formed of two domains, βαβαβα from residues 1 to 59 (domain 1) and ββα from residues 76 to 108 (domain 2) (Katti, S. K., LeMaster, D. M. & Eklund, H. (1990) *J. Mol. Biol.*

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212, 167-184. The DEZYMER solution (Figure 7), which is located between the central β-sheet (Cys24, β-strand 2; Cys55, β-strand 3) and one α-helix (Cys42, α-helix 2; Cys99, α-helix 4) from each domain, is buried approximately 8-9 Å from the surface. All peptide amide NH groups in the vicinity of the designed site participate in secondary structure interactions in this region and are unavailable for hydrogen bonding to the cluster in the model. Finally, the cluster site is buried in the interior of the Trx host and surrounded by a hydrophobic shell generated by Ile41, Ile45, Ala46, Leu53, Leu78, Leu94 and Leu103.

The DEZYMER algorithm is based on the premise that the mutations introduced are conservative and maintain the overall protein fold of the host scaffold (inverse folding constraint). This assumption was tested by comparing the *in vivo* biological properties of Trx-Fe_4S_4 and wild-type Trx. The cluster binding site was introduced into a region of Trx that is located near the putative protein-protein interaction surface required for the growth of filamentous phages, thereby allowing a test of whether the mutations in Trx-Fe_4S_4 affect the original functions of Trx. Trx-Fe_4S_4 exhibited *full activity*, thereby providing strong evidence that the designed protein adopts a wild-type conformation or at least maintains the essential structural features of the Trx surface that are necessary for this *in vivo* activity. Furthermore, both the CD spectra (apo) and hydrodynamic (apo, holo) properties of Trx-Fe_4S_4 are equivalent to the Trx host, offering both genetic and physical data suggesting that the overall structure of Trx-Fe_4S_4 is essentially indistinguishable from Trx. The CD spectrum of holo Trx-Fe_4S_4 is dominated by signals from the cluster is relatively uninformative regarding the protein fold. Complete structural determinations of apo and holo Trx-Fe_4S_4 are ultimately required to assess the success of the design.

Evidence for cuboidal cluster incorporation into the designed site comes from both optical and EPR studies. The excellent stability of the Trx-Fe_4S_4 chromophore in the absence of free thiols, the bathochromic shift in the cluster chromophore upon protein reconstitution, coupled with an EPR silent resting state and the observation of a paramagnetic cluster upon cluster oxidation all indicate the presence of a protected cuboidal {Fe_4S_4} cluster. Determination of the expected Fe:S^{2-}:Trx, the similarity of the Trx-Fe_4S_4 chromophore to that of known $Fe_4S_4Cys_4$ proteins, and its dissimilarity to

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other ($Fe_2S_2Cys_4$, $FeCys$) cluster types argues against the possibility of cluster fragmentation accompanied by iron binding.

     Ferricyanide oxidation also places a limit on the redox properties of the cluster, consistent with its HiPIP-like properties. The oxidized Trx-Fe_4S_4 cluster is only

5   transiently stable, as evidenced by rapid bleaching of the visible absorbance upon ferricyanide addition, and the disappearance of the $g_{avg} > 2$ EPR signal upon prolonged incubation at 4 °C. Such cluster degradation has been reported for HiPIPs and is believed to produce reducing equivalents (Fe^{2+}, S^{2-}, and CysSH) (Bian, S., *et al.* (1996) *Biochemistry*, 35, 14544-552). While these factors complicate the determination of a

10 redox potential, the observation of complete oxidation upon addition of >7 equivalents of potassium ferricyanide, and incomplete oxidation at lower concentrations imposes a lower bound of +300 mV *vs.* NHE on the redox potential of Trx-Fe_4S_4, based on the Nernst equation and the {ferricyanide}/{ferrocyanide} ratio. This potential is within the range of redox potentials observed for HiPIPs ($E^o = +100$ mV to +450 mV).

15   Thus, in key aspects of the present invention, the present inventors have successfully created a cluster of the correct topological type, and furthermore have determined that holo Trx-Fe_4S_4 maintains the folded structure of its unmodified host protein, and that incorporation of a cuboidal {$Fe_4S_4(Cys)_4$}$^{2-/1-}$ cluster in the hydrophobic interior of Trx is sufficient to generate a HiPIP-like metalloprotein. Therefore, it has been

20 found that the DEZYMER algorithm makes valid predictions based on simple geometric principles without explicit considerations of binding thermodynamics or protein dynamics. The present inventors have demonstrated that these approximations can have sufficient predictive power to redesign the hydrophobic interior of a protein and introduce a {Fe_4S_4} cluster.

25   Unexpectedly, the designed site does not follow any of the consensus sequences observed in proteins containing cuboidal {Fe_4S_4} clusters (Figure 11) (Johnson, M. K. (1994) in *Iron-Sulfur Proteins*, ed. King, R. B. (John Wiley & Sons, Oxford), Vol. 4, pp. 1896-1915). The consensus sequence for coordination of a {Fe_4S_4} cluster in bacterial Fds is Cys-X_2-Cys-X_2-Cys with a more remote Cys residue supplying the fourth ligating

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sulfur center. Similarly, in 8Fe Fds, the consensus sequence involves two $Cys-X_2-Cys-X_2-Cys-X_3-Cys-Pro$ sequences with each cluster coordinated by three Cys residues from one group and a more remote Cys residue. Previously, a ferredoxin-like cluster was made from synthetic polypeptides derived from the consensus sequence of natural ferredoxins

5   (Nakamura, A. & Ueyama, N. (1994) in *Encyclopedia of Inorganic Chemistry* ed. King, R. B. (Wiley, New York), Vol. 4, pp. 1883-1896; Gibney, B. R., Mulholland, S. E., Rabanal, F. & Dutton, P. L. (1996) *Proc. Nat'l Acad. Sci. USA* **93**, 15041-15046). Different arrangements of coordinated Cys residues are also found for the Fe_4S_4 clusters of HiPIP proteins ($Cys-X_2-Cys-X_{16}-Cys-X_{13}-Cys$). The coordinating Cys residues in Trx-

10   Fe_4S_4 are found in the pattern $Cys-X_{18}-Cys-X_{13}-Cys-X_{44}-Cys$ which does not follow the standard Fd or HiPIP patterns for creating the ligand sphere for the $\{Fe_4S_4\}$ cluster. The existence of the Cys residues at locations widely separated in primary sequence but appropriately positioned in the three dimensional space of the folded scaffold protein offers the opportunity to construct clusters in different secondary structural environments.

15   Simulation studies have shown that correlating variations of redox potential with protein environment requires assessing the Coulombic interaction of the cluster with the protein (the major determinant), the polarizability of the protein, and the interaction of the cluster and protein with solvent (Stephens, P. J., Jollie, D. R. & Warshel, A. (1996) *Chem. Rev.* **96**, 2491-2513). These results suggest that the folding of the polypeptide in the

20   vicinity of the cluster (proximity and orientation of cluster charge-amide dipole interaction) is a major factor in fixing the redox potential; sidechain groups are proposed to be of secondary importance. The DEZYMER algorithm places no *a priori* constraint on the choice of the secondary structural environment in which the new place is incorporated, offering the opportunity to test this hypothesis. The fact that the orientation of the amide

25   dipoles in the model of $Trx-Fe_4S_4$ are fixed by the folded structure of Trx and not optimized for the presence of the incorporated cluster is consistent with the HiPIP-like redox properties of $Trx-Fe_4S_4$ (Stephens, P. J., *supra*). Finally, the geometry of the designed cluster is such that neighboring hydrophobic side-chains can be mutated to

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introduce hydrogen bonding or charged residues in order to systematically probe the role

of these determinants in the electronic properties and redox potential of the cluster.

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What is Claimed is:

1.   A method of preparing a functional metalloprotein containing at least one metal
binding site, said site consisting of at least one metal ion and ligands associated
therewith and having a calculated volume equal to the sums of the volumes of the

5   metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination
sphere, atoms bonded directly to the metal ion and in a second coordination
sphere, all atoms bonded directly and indirectly to the atoms of the first
coordination sphere between the metal ion(s) and the backbone of the protein,
which comprises:

10   (A) selecting a host protein having a tertiary structure characterized with
a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein
which collectively form the first and second coordination spheres of the
metal binding site and result in overpacking of the tertiary structure to an

15   extent of less than about 10% in mean atomic displacement, to provide a
designed apoprotein, wherein said determining comprises:

(1) representing the structure of the host protein as a three-
dimensional atomic coordinate array, wherein side chainsare
removed leaving the protein backbone;

20   (2) defining the first coordination sphere of the metal binding site,
comprising metal ion(s), bond lengths, bond angles, dihedral angles
and exogenous ligands;

(3) identifying volume elements in the host protein excluding the
backbone of the protein equal to between about 90% and 110% of

25   the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume
elements, thereby generating a set of primary sequence mutations;

(5) calculating the volume change resulting from each set of
primary sequence mutations;

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(6) selecting the set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein

5   containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed apoprotein;

10   (D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

(E) overexpressing the designed apoprotein in the host organism; and

(F) either (i)   purifying the overexpressed designed apoprotein, and reconstituting it with metal ion(s) or metal cluster under

15   suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein;

20   or

(ii)   incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

25   2.   The method of claim 1 wherein the functional metalloprotein contains one metal binding site.

3.   The method of claim 1 wherein the metal binding site comprises a cuboidal iron-sulfur cluster.

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4.   The method of claim 1 wherein the metal binding site comprises a mononuclear iron(II/III)-histidine.

5   5.   The method of claim 1 wherein primary sequence mutations are determined using an algorithm selected from the group consisting of DEZYMER, PROPAK and METALSEARCH.

6.   The method of claim 1 wherein the structure of the host protein is obtained as a
10   three-dimensional atomic coordinate array from X-ray crystallography or nuclear magnetic resonance spectroscopy.

7. The method of claim 1 wherein the host protein is a physiological plasma protein, thioredoxin or human serum albumin.
15

8. The method of claim 1, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

20   (2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino
25   acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

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(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein.

9.   A functional metalloprotein containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and in a second coordination sphere, all atoms bonded directly and indirectly

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to the atoms of the first coordination sphere between the metal ion(s) and the backbone
of the protein, which comprises:

(A) selecting a host protein having a tertiary structure characterized with
a resolution of less than or equal to about 3.0Å;

5   (B) determining a set of primary sequence mutations in the host protein
which collectively form the first and second coordination spheres of the
metal binding site and result in overpacking of the tertiary structure to an
extent of less than about 10% in mean atomic displacement, to provide a
designed apoprotein, wherein said determining comprises:

10   (1) representing the structure of the host protein as a three-
dimensional atomic coordinate array;

(2) defining the first and second coordination spheres of the metal
binding site, comprising metal ion(s), bond lengths, bond angles,
dihedral angles and exogenous ligands;

15   (3) identifying volume elements in the host protein excluding the
backbone of the protein equal to between about 90% and 110% of
the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume
elements, thereby generating a set of primary sequence mutations;

20   (5) calculating the volume change resulting from each set of
primary sequence mutations;

(6) selecting the set of primary sequence mutations wherein the
volume change corresponding thereto is not more than between
about 90% and 110% of the volume of the metal binding site and

25   wherein the tertiary structure of the resulting metalloprotein
containing such set of mutations is overpacked to an extent of less
than about 10% in mean atomic displacement, thereby providing
the designed apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence

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mutations determined in step (B)(6) for the designed apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed apoprotein;

(E) overexpressing the designed apoprotein in the host organism; and

(F) either (i) purifying the overexpressed designed apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed apoprotein, treating the partially denatured designed apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii) incubating the metal ion(s) or metal cluster with the designed apoprotein and purifying directly an intact functional metalloprotein.

10. The functional metalloprotein of claim 9, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the

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protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

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a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii overlap, the residues are repacked; and

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b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and

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110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not

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more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed apoprotein.

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11.   The functional metalloprotein of claim 9 wherein said protein contains one metal ion binding site.

12.   The functional metalloprotein of claim 9 wherein primary sequence mutations are

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determined using an algorithm selected from the group consisting of DEZYMER, PROPAK and METALSEARCH.

13.   The functional metalloprotein of claim 9 wherein the structure of the host protein
5   is obtained as a three-dimensional atomic coordinate array from X-ray crystallography or nuclear magnetic resonance spectroscopy.

14. The functional metalloprotein of claim 9 wherein the host protein is a physiological plasma protein, thioredoxin or human serum albumin.
10

15.   A synthetic superoxide dismutase containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to
15   the metal ion and in a second coordination sphere, all atoms bonded directly and indirectly to the atoms of the first coordination sphere between the metal ion(s) and the backbone of the synthetic superoxide dismutase, said synthetic superoxide dismutase being prepared by:

      (A) selecting a host protein having a tertiary structure characterized with
20   a resolution of less than or equal to about 3.0Å;

      (B) determining a set of primary sequence mutations in the host protein which collectively form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a
25   designed superoxide dismutase apoprotein, wherein said determining comprises:

            (1) representing the structure of the host protein as a three-dimensional atomic coordinate array;

            (2) defining the first and second coordination spheres of the metal

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binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) identifying volume elements in the host protein excluding the backbone of the protein equal to between about 90% and 110% of the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating a set of primary sequence mutations;

(5) calculating the volume change resulting from each set of primary sequence mutations;

(6) selecting the set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed superoxide dismutase apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed superoxide dismutase apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed superoxide dismutase apoprotein;

(E) overexpressing the designed superoxide dismutase apoprotein in the host organism; and

(F) either (i)   purifying the overexpressed designed superoxide dismutase apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional metalloprotein, wherein said reconstituting optionally comprises partially denaturing the designed superoxide dismutase apoprotein, treating the partially denatured

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designed superoxide dismutase apoprotein with the metal ion(s) or metal cluster and concurrently refolding the metalloprotein; or

(ii)   incubating the metal ion(s) or metal cluster with the designed superoxide dismutase apoprotein and purifying directly an intact functional metalloprotein.

16. The synthetic superoxide dismutase of claim 15, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii

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overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

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(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence

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and residue rotamers are varied, such that sequence mutations are selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting synthetic superoxide dismutase containing such set of mutations is

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overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed synthetic superoxide dismutase apoprotein.

17.   The synthetic superoxide dismutase of claim 15 wherein said superoxide dismutase

20   contains one metal ion binding site.

18.   The synthetic superoxide dismutase of claim 15 wherein primary sequence mutations are determined using an algorithm selected from the group consisting of DEZYMER, PROPAK and METALSEARCH.

25

19.   The synthetic superoxide dismutase of claim 15 wherein the structure of the host protein is obtained as a three-dimensional atomic coordinate array from X-ray crystallography or nuclear magnetic resonance spectroscopy.

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20. The synthetic superoxide dismutase of claim 15 wherein the host protein is a physiological plasma protein, thioredoxin or human serum albumin.

5   21.   A synthetic monooxidase containing at least one metal binding site, said site consisting of at least one metal ion and ligands associated therewith and having a calculated volume equal to the sums of the volumes of the metal ion(s) and all of the ligands, wherein said ligands are, in a first coordination sphere, atoms bonded directly to the metal ion and, in a second coordination sphere, all atoms bonded directly and indirectly

10   to the atoms of the first coordination sphere between the metal ion(s) and the backbone of the synthetic monooxygenase said synthetic monooxygenase being prepared by:

(A) selecting a host protein having a tertiary structure characterized with a resolution of less than or equal to about 3.0Å;

(B) determining a set of primary sequence mutations in the host protein

15   which collectively form the first and second coordination spheres of the metal binding site and result in overpacking of the tertiary structure to an extent of less than about 10% in mean atomic displacement, to provide a designed monooxygenase apoprotein, wherein said determining comprises:

(1) representing the structure of the host protein as a three-

20   dimensional atomic coordinate array;

(2) defining the first and second coordination spheres of the metal binding site, comprising metal ion(s), bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) identifying volume elements in the host protein excluding the

25   backbone of the protein equal to between about 90% and 110% of the volume of the metal binding site;

(4) combinatorially replacing sequence residues in said volume elements, thereby generating a set of primary sequence mutations;

(5) calculating the volume change resulting from each set of

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primary sequence mutations;

(6) selecting set of primary sequence mutations wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting metalloprotein containing such set of mutations is overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed monooxygenase apoprotein;

(C) preparing a nucleotide sequence corresponding to the set of sequence mutations determined in step (B)(6) for the designed monooxygenase apoprotein;

(D) transfecting a host organism with the nucleotide sequence of the designed monooxygenase apoprotein;

(E) overexpressing the designed monooxygenase apoprotein in the host organism; and

(F) either (i) purifying the overexpressed designed monooxygenase apoprotein, and reconstituting it with metal ion(s) or metal cluster under suitable conditions to form the functional monoxygenase, wherein said reconstituting optionally comprises partially denaturing the designed monooxygenase apoprotein, treating the partially denatured designed monooxygenase apoprotein with the metal ion(s) or metal cluster and concurrently refolding the monooxygenase; or

(ii) incubating the metal ion(s) or metal cluster with the designed superoxide dismutase apoprotein and purifying directly an intact functional monooxygenase.

22. The synthetic monooxidase of claim 21, wherein said determining comprises:

(1) representing the structure of the host protein as a three-dimensional

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atomic coordinate array, wherein side chains are removed leaving the protein backbone;

(2) defining the first coordination sphere of the metal binding site as the metal ion(s) subject to a set of geometrical constraints, comprising bond lengths, bond angles, dihedral angles and exogenous ligands;

(3) combinatorially searching said three-dimensional coordinate array, said geometrical constraints and a library of side chain rotamers for each amino acid to identify sets of sequence residues, rotamers and ligand positions for each metal binding site;

(4) constructing a close-packed complementary surface between the first coordination sphere of the metal binding site and the backbone of the protein, wherein said constructing comprises:

(i) combinatorially replacing all original residues not changed by placement of the metal binding site(s);

(ii) identifying residues to be repacked, comprising:

a) determining whether the residues make steric contact with any portion of the first coordination sphere, wherein van der Waals' radii of atoms comprising the residues and the first coordination sphere overlap, such that if said radii overlap, the residues are repacked; and

b) determining whether a replaced residue creates a void by calculating a probe-accessible surface, wherein if a void is created, the replaced residue is repacked;

(3) calculating volume elements in the host protein to be filled excluding the backbone of the protein equal to between 90% and 110% of the volume of the metal binding site, said volume elements comprising a sum of replaced residue volumes;

(4) executing a repacking algorithm, wherein the primary sequence and residue rotamers are varied, such that sequence mutations are

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selected wherein the volume change corresponding thereto is not more than between about 90% and 110% of the volume of the metal binding site and wherein the tertiary structure of the resulting synthetic monooxidase containing such set of mutations is

5   overpacked to an extent of less than about 10% in mean atomic displacement, thereby providing the designed synthetic monooxidase apoprotein.

23.   The synthetic monooxidase of claim 21 wherein said monooxidase contains one

10   metal ion binding site.

24.   The synthetic monooxidase of claim 21 wherein primary sequence mutations are determined using an algorithm selected from the group consisting of DEZYMER, PROPAK and METALSEARCH.

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25.   The synthetic monooxidase of claim 21 wherein the structure of the host protein is obtained as a three-dimensional atomic coordinate array from X-ray crystallography or nuclear magnetic resonance spectroscopy.

20   26. The synthetic monooxidase of claim 21 wherein the host protein is a physiological plasma protein, thioredoxin or human serum albumin.

27. A method of oxidizing an alkane which comprises treating the alkane with a functional metalloprotein containing a metal binding site wherein the metal binding site is a selected

25   from the group consisting of a $Mo(=O)_2$ complex and a mononuclear iron center together with a cubic iron-sulfur cluster under conditions suitable to form an oxidized alkane.

28. The method of claim 27 wherein the functional metalloprotein is selected from the group consisting of ferredoxin, human serum albumin, a catalytic antibody, a tyrosinase and a superoxide dismutase.

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29. A method of treating a subject suffering from ischemia which comprises administering to the subject a functional metalloprotein prepared from a host protein containing a metal binding site wherein the protein has superoxide dismutase activity in a therapeutically effective amount.

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30. The method of claim 29 wherein the host protein is a human serum albumin, and the metal ion binding site comprises Fe(II/III) and a histidine ligand.

31. A method of treating a subject suffering from reperfusion type tissue damage which
15 comprises administering to the subject a functional metalloprotein prepared from a host protein containing a metal binding site wherein the protein has superoxide dismutase activity in a therapeutically effective amount.

32. The method of claim 31 wherein the host protein is a human serum albumin, and the
20 metal ion binding site comprises Fe(II/III) and a histidine ligand.